IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

BELLGRAU et al.

Serial No.: 10/825,282

Filed: April 14, 204

Atty. File No.: 3921-1-1-1-1

For: VIRAL VECTORS ENCODING APOPTOSIS-INDUCING PROTEINS AND METHODS FOR MAKING AND USING THE SAME

Group Art Unit: 1633

Examiner: Kaushal, S.

DECLARATION OF DONALD BELLGRAU, RICHARD C. DUKE, AND JEROME B. SCHAACK (37 CFR § 1.131)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

We, Donald Bellgrau, Richard C. Duke and Jerome B. Schaack, each declare as follows:

1. I am a co-inventor of the above-referenced patent application and am familiar with the application.

2. This Declaration under 37 CFR 1.131 is being submitted in conjunction with an Amendment and Response to an Office Action having a mailing date of April 18, 2007.

3. This Declaration provides factual evidence that the invention as claimed in the above-identified patent application was conceived of and reduced to practice at least prior to the publication date of Shinoura et al., *Human Gene Therapy* 9(18):2683-2689, which is December 10, 1998. All acts relied upon to establish the dates of conception and actual reduction to practice were carried out in the United States.

Conception and Reduction to Practice

As evidence of conception and actual reduction to practice of the claimed invention by a date prior to at least December 10, 1998, enclosed as Exhibit A is a manuscript that discloses completed experiments that were performed in the present inventors' laboratories and that describe the presently claimed invention. This manuscript was prepared by a date prior to at least December 10, 1998 and accordingly, the

experiments described therein were completed by a date prior to at least December 10, 1998.

Specifically, this manuscript sets forth and exemplifies the basic concept as claimed in the present invention, which is the method of propagating a recombinant viral vector encoding an apoptosis-inducing protein, as well as recombinant production cells used in the method and a recombinant vector propagated by the method. The manuscript identifies the problem to be solved (*i.e.*, sensitivity of the recombinant production cell to the apoptosis-inducing protein), and the novel solution to the problem (i.e., coexpression of an apoptosis-inducing protein and an apoptosis-inhibiting protein in the production cell).

The manuscript describes the following exemplary experiments that are relevant to the presently claimed invention: (1) construction of a viral vector encoding Fas ligand (an exemplary apoptosis-inducing protein) (see, *e.g.*, pages 6-7); (2) successful propagation of the viral vector using the claimed method, specifically, by coexpression in a host cell with a recombinant nucleic acid molecule encoding an exemplary apoptosis-inhibiting protein, CrmA (see, *e.g.*, page 12 and Fig. 2); and (3) a demonstration that the viral vector produced by the claimed method operates for its intended purpose by a showing that the vector efficiently transfects a cell so that Fas ligand expression is achieved (see, *e.g.*, page 13 and Table II), which is particularly relevant to Claims 1-43. The manuscript also describes experiments demonstrating that apoptosis is induced by the expression of Fas ligand in tumor cells *in vitro* and *in vivo* (see, *e.g.*, pages 13-15 and Figs. 3 and 4 and Table III), which is particularly relevant to Claims 45-50, 54, 55, 64, and 65.

These experiments therefore demonstrate the practice of the claimed method to propagate a recombinant viral vector, as well as production of isolated cells used in the method and a vector produced using the method. The experiments also establish that the method, cells and vector operated for their intended purpose both *in vitro* and *in vivo*.

This evidence is believed to be sufficient to establish conception and an actual reduction to practice of the claimed invention.

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10/12/07
Date

Donald Bellgrau

Date

Richard C. Duke

Date

Jerome B. Schaack

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RCD
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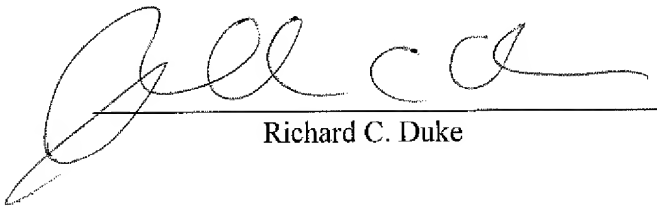
10-16-07 Date	 Richard C. Duke
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Date	Donald Bellgrau
Date	Richard C. Duke
10/15/07 Date	Jerome B. Schaack

EXHIBIT A

Abstract

Recent evidence has suggested that the Fas apoptotic signaling pathway is involved in the regulation of epithelial cell turnover in the prostate. Several laboratories have also reported on the apoptotic potentials of human prostate cancer (PC) cell lines in response to Fas signaling. Much of this data, however, utilized agonistic anti-Fas antibodies to activate Fas receptors. In the current study, we re-evaluated the apoptotic potentials of 7 human PC cell lines using the natural Fas ligand (FasL) in place of agonistic antibody. First, PC cell lines were tested in a standard cytotoxicity assay with a transfected cell line that stably expresses human FasL. Next we developed an adenoviral expression system to analyze the effects of FasL when expressed internally by the PC cell lines. This system was optimized by the use of 293 cells that were transfected with a crmA cDNA construct. Together, these data suggest that the apoptotic potentials of these cell lines were greatly underestimated in previous studies with agonistic anti-Fas antibodies. Lastly, adenoviral expression of FasL prevented growth and induced regression of two PC cell lines in *Nu/Nu* mice. These preliminary *in vivo* results suggest potential for the use of adenovirus-FasL as a gene therapy approach for PC.

Introduction

Fas is a transmembrane glycoprotein that is related to the receptors for Tumor Necrosis Factor and Nerve Growth Factor (Itoh 1991; Oehm 1992). Upon binding to Fas Ligand (FasL) (Suda 1993), the Fas receptor initiates a complex signal transduction pathway that, in sensitive cell types, ultimately ends in apoptotic cell death (Baker 1996). The Fas/FasL pathway is probably best recognized in the immune system for its role in the downregulation of expanded clonal T lymphocyte populations. In this system Fas is upregulated within a few hours of T cell activation (Miyawaki 1992). Several days later, FasL is upregulated (Owen-Schaub 1992; Suda 1993) and the majority of cells in the clonal population undergo apoptosis, allowing the immune system to return to its normal resting size and repertoire.

In addition to regulating the immune response, Fas and FasL are likely to play a role in other systems as well. For example, the testes and placenta, both of which are known to be immune-privileged tissues, express high levels of FasL (Xerri 1997). Additionally, Fas and FasL have been found to be coexpressed in a few epithelial tissues that are marked by apoptotic cell turnover, such as the uterus and prostate (Leithauser 1993; French 1996; Xerri 1997). Both of these tissues are steroid-dependent and undergo apoptosis within 24-48 hours after hormone depletion (Isaacs 1992; Rotello 1992). Interestingly, the apoptosis that occurs in response to steroid depletion has recently been shown to require sufficient Fas expression (Suzuki 1996; Suzuki 1996). These data support a role for the Fas signaling pathway in the normal renewal of the uterine and prostatic epithelium.

In addition to the *in vivo* data above, several laboratories have recently demonstrated a potential role for Fas-mediated apoptosis in human prostate cancer (PC) cell lines(Rokhlin 1997; Uslu 1997; Hedlund 1998). Although Fas expression has proven to be a common feature of the cell lines studied, contradictory results were

reported with regards to their apoptotic potentials (Rokhlin 1997; Uslu 1997; Hedlund 1998). This may possibly owe to the use of different agonistic anti-Fas antibodies among laboratories, or to the different experimental conditions that were employed.

Preliminary data in our laboratory have suggested that the apoptotic potential of at least one PC cell line is markedly increased when the cells are coincubated with a transfected leukemia cell line that stably expresses human FasL. For this reason, we began re-evaluating the sensitivities of the seven PC cell lines used previously (Hedlund 1998) to determine if the use of anti-Fas IgM antibodies commonly under-estimated or mis-estimated the apoptotic potentials of these cells. We next developed an adenoviral method of gene transduction to study the effects of FasL when expressed internally by the PC cells. This system was optimized by the use of 293 cells that were transfected with a crmA cDNA construct (Tewari 1995; Tewari 1995) to provide them with resistance to Fas-mediated apoptosis, and increase the efficiency of virus production. Lastly, we present *in vivo* evidence for a potential gene therapy approach for PC using adenoviral-FasL expression.

Materials and Methods

Nontransfected Cell Cultures. The human PC cell lines were kindly provided by the following laboratories: LNCaP (Horoszewicz 1980; Horoszewicz 1983), Dr. J. Horoszewicz, Roswell Park Memorial Institute, Buffalo, NY; ALVA-31 (Loop 1993), Dr. Steven Loop, American Lake VAMC, Tacoma, WA; TSU-Pr1 (Iizumi 1987), Dr. J. Isaacs, The Johns Hopkins Cancer Center, Baltimore, MD; JCA-1 (Muraki 1990), Dr. J. Chiao, New York Medical College, Valhalla, NY; PPC-1 (Brothman 1989), Dr. A. Brothman, University of Utah, Salt Lake City, UT. The cell lines PC-3 (Kaighn 1979)and DU 145 (Stone 1978) were obtained from American Type Culture Collection (Rockville, MD). The authenticities of each of these cell lines have been confirmed by the Cytogenetics Core laboratory at the University of Colorado Health Sciences Center (UCHSC), Denver, CO.

The following cell lines were also purchased from American Type Culture Collection: K562, an erythroid leukemia cell line (Lozzio 1975; Andersson 1979); 293, a human embryonic kidney cell line transformed by the E1 region of the adenovirus 5 genome (Graham 1977). All cultures were maintained in RPMI 1640 (Gibco, Grand Island, NY) with 7.5% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), and an additional supplement of 2 mM L-glutamine (Gibco). Cell cultures were incubated at 37° C in 95% air and 5% CO_2.

Stably-Transfected Cell Lines. 1) K562-FasL: K562 cells were cotransfected with plasmids encoding human FasL and neomycin phosphotransferase cDNAs. Geneticin-resistant cells were cloned by limiting dilution, and were assayed for their ability to kill Fas-bearing L1210 leukemia cells. In the current experiments, clone G10 was utilized (Duke 1998). Further characterization of these clones is the focus of a separate

manuscript (S. Meech and R. Duke, manuscript in preparation). 293-crmA: a modified calcium phosphate precipitation technique (Jordan 1996) was used to transfect 293 cells with a pcDNA3-*crm*A construct (Tewari 1995; Tewari 1995) generously provided by Dr. V. Dixit (Genentech Inc., South San Francisco, CA). Stable transfectants were selected with 400 μg/ml active Geneticin (Gibco), but were not cloned. Functional expression of crmA was assessed by transient transfection of the cells with the CMV-FasL construct used for the K562-FasL clones. While 293-crmA cells remained viable, the parental 293 cells became extensively apoptotic within 24 h (data not shown). 3) L1210-Fas (Rouvier 1993): this Fas-transfected mouse lymphocytic leukemia cell line expresses high levels of mFas. This clone was kindly provided by Dr. P. Golstein (Marseilles, France). All transfected cell lines were routinely cultured in the presence of Geneticin, using the medium and conditions described for nontransfected cells.

Adenovirus Constructs and Viral Amplification. The murine FasL cDNA was generated by RT-PCR (Bellgrau 1995) and was inserted in the sense orientation into pACCMV (Gomez-Foix 1992). This plasmid encodes the left end of the Ad5 chromosome and contains a CMV immediate early promoter expression cassette in place of the E1 region (Gomez-Foix 1992). Ad5*dl*327CMV-eGFP encodes a humanized, enhanced, red-shifted jellyfish green fluorescent protein (Zhang 1996) (Clontech Laboratories, Palo Alto, CA) under the control of the CMV immediate early promoter. Its construction is described elsewhere (J. Schaack et al., manuscript in preparation). Ad5*dl*327$_{Bst}$β-gal-TP complex was prepared by banding purified Ad5*dl*327$_{Bst}$β-gal virions in 4 M guanidine-HCl (Sigma Chemical Company, St. Louis, MO), 2.8 M cesium chloride (Baxter, McGraw Park, IL) (Jones 1978). The gradient was fractionated and fractions containing Ad5*dl*327$_{Bst}$β-gal-TP complex were collected and dialyzed versus H_2O. The DNA-TP complex was then digested with BstBI (New England Biolabs, Berverly, MA), which cleaves uniquely 3' of the LacZ coding sequence (Schaack 1995). The pACCMV-

mFasL DNA was then mixed with BstBI-digested Ad5*dl*327$_{BstB}$-gal-TP complex and was used to transfect 293-crmA cells using $Ca_3(PO_4)_2$ precipitation (Jordan 1996). After 5 hr, the precipitate was removed and fresh medium was added. The transfected cells were incubated until the development of a strong cytopathic effect. The cells were then harvested and freeze-thawed to release virus. Dilutions of the cell lysate were used to infect 293-crmA cells, which were then overlaid with medium containing Noble agar (Difco, Detroit, MI). After plaques developed, the cells were stained with neutral red (Baxter) and X-gal (Boehringer Mannheim, Indianapolis, IN). Plaques that were clear in the presence of X-gal, and thus likely to be recombinants (Schaack 1995), were picked and grown in 293-crmA cells. Lysates of the plaque-infected cells were used to infect 293-crmA cells. Viral DNAs were isolated (Hardy 1997) and restriction analysis used to determine whether the recombinant viruses contained the FasL expression cassette. Viruses that contained the FasL gene were grown for functional testing.

Adenovirus Transduction of PC Cells and Growth Assays. Cell monolayers were trypsinized and washed once with standard growth medium. For each cell line $3.2x10^5$ cells (negative control) or $2.4x10^5$ cells (adeno-GFP and adeno-FasL) were placed into each of 3 conical Eppendorf tubes. Cells were pelleted by centrifugation, and supernatants were removed by aspiration. The pellets were resuspended in 500 µl of either plain medium (negative control), or 500 µl of medium containing approximately 100 pfu/cell of the adeno-FasL or adeno-GFP viral lysates described above. Cells were incubated for 1 h in a 37° C water bath with periodic mixing and were then washed twice with medium. The final cell pellets were resuspended in 16 ml medium (control cells) or 12 ml medium (adFasL and adGFP cells. Each sample was then aliquotted into quadruplicate wells (1 ml each) of three 24-well tissue culture plates. To quantitate the amount of DNA in the initial number of cells plated (i.e. Day 0), 1 ml aliquots of the negative control were placed in 4 Eppendorf tubes. Cells were pelleted, supernatant

was aspirated, and the cell pellets were lysed in 0.25 ml 0.5 M NaOH. Cell lysates were then frozen at -20° C until all time points were collected. The remaining cultures were incubated at 37° C. Tissue culture medium was replaced every 48 h, and cells were harvested at the designated time points by aspirating the medium and lysing the monolayers in 0.5 M NaOH (0.25 ml/well), and freezing at -20° C. The DNA contents of the monolayers were quantitated by Hoechst 33258 fluorescence (Labarca 1980; Hedlund 1994) using a Dynex Fluorolite 1000 fluorescence plate reader (Dynex Technologies, Inc., Chantilly, VA). Mean DNA concentrations were calculated ± standard error.

^{51}Cr-Release Assay for Cytotoxicity . The original assay (Duke 1983) was modified as follows: PC cell lines were removed from the culture flasks by incubating at 37° C in a trypsin-free chelating solution (135 mM NaCl, 5 mM KCl, 20 mM HEPES, and 1.5 mM EDTA, pH 7.4). For each target cell line, 10^6 cells were pelleted and resuspended in 1 ml RPMI 1640 supplemented with 7.5% FBS and 10 mM HEPES buffer. Cells were labeled with 100 µCi ^{51}Cr (specific activity 216 mCi/mg Cr, ICN Pharmaceuticals Inc., Irvine, CA) in this medium for 1 hr at 37° C. Excess ^{51}Cr was removed by washing twice in 10 ml medium, incubating for 1 hr in fresh medium at 37° C, and washing again. 5000 radiolabeled target cells in 100 µl were placed in each well of a round bottom 96-well tissue culture plate. Effector cells (either K562-FasL or K562-vector transfectants) were added at 10:1, 1:1, or 0.1:1 ratios to the target cells, also in a volume of 100 µl per well. Maximum lysis was determined by adding 100 µl 1% Triton X-100 instead of effector cells. Spontaneous lysis was estimated by using 100 µl medium instead of effector cells. All conditions were carried out in triplicate. Cells were incubated for 16-20 h at 37° C. The 96-well plates were centrifuged briefly at 500 RPM to pellet floating cells. Supernatants (100 µl per well) were transferred to separate tubes and radioactivity was quantitated using a gamma counter. Spontaneous release of

^{51}Cr was subtracted from each value, and cell lysis was presented as a percent of maximum lysis. Each value represents the mean of triplicate samples ± standard deviation.

Fas Receptor Quantitation. Cell monolayers were harvested at 50-60% confluency using the trypsin-free chelating solution described for ^{51}Cr-release experiments. The cells were washed once with PBS, pH 7.4, and once with the blocking solution (PBS with 5% goat serum and 0.1% sodium azide). $5x10^5$ cells were placed in each of 2 Eppendorf tubes for each cell line. Cells were pelleted again and were resuspended in 200 µl of blocking solution containing either 2 µg/ml mouse anti-human Fas-IgG1 that is directly conjugated to FITC (clone DX2, PharMingen, San Diego, CA), or 2 µg/ml mouse anti-*Aspergillus* niger glucose oxidase IgG1-FITC (clone DAK-G01, Dako). Samples were gently vortexed and were incubated at room temperature for 15 min, protected from light. Cells were washed once with PBS, were fixed with 1% formaldehyde in PBS for 5 min, and were washed again. Each sample was then resuspended in 0.5 ml PBS for fluorescence analysis on a Coulter XL flow cytometer (Coulter Corp., Hialeah, FL).

Prostate Tumor Growth in Athymic Nude Mice. ALVA-31 and TSU-Pr1 cells were transduced with the adenovirus constructs as described for the growth assays, with the exception that viral titers were reduced to 10 pfu/cell for practicality. Control cells were incubated in tissue culture medium in the absence of virus. Cells were then washed twice with tissue culture medium and once with PBS. Cell pellets were resuspended in PBS to yield a concentration of $3x10^6$ cells per 100 µl (TSU-Pr1) or $2x10^6$ cells per 100 µl (ALVA-31). For each PC cell line, 4 male *Nu/Nu* mice (National Cancer Institute, Bethesda, MD), ages 6-8 weeks old, were injected intradermally with 100 µl of the cell suspensions in a total of 5 sites per mouse including both shoulders, both hips,

and the center of the back. Each mouse, therefore, harbored 3 control tumors, 1 adeno-FasL, and 1 adeno-GFP tumor.

When control tumors were approximately 0.5 cm in size (approximately 10 days later), two control tumors per mouse were injected in the centers with 50 µl free adeno-FasL or adeno-GFP virus ($5x10^8$ pfu/ml) to determine if regression or rejection could be initiated in an already established tumor. One mouse was then sacrificed 24 h later to histologically examine the tumors. Tumor sizes were measured using calipers. Two to six weeks after the start of the experiment, the remaining mice were sacrificed and the excized tumors were fixed in formalin and embedded in paraffin. Tissue sections were then stained with hematoxylin and eosin (Histology Laboratory, Dept. Surgical Pathology, UCHSC). Histologic analyses were completed with the assistance of two objective and trained Pathologists, Drs. John Ryder and Rosina DeCampo (Dept. Pathology, UCHSC).

Results and Discussion

PC Cell Lines Show Enhanced Sensitivity To hFasL Versus Agonistic Antibody. Target cell lines were labeled with ^{51}Cr and were co-incubated for 18 h with various ratios of the FasL-expressing effector cells (K562-FasL transfectants). Each of the 8 target cell lines were tested simultaneously in the cytotoxicity assays to allow for the direct comparison of their apoptotic potentials. The cell line L1210-Fas served as a positive control since it is known to be highly sensitive to Fas-mediated death (Rouvier 1993). As shown in Fig. 1A, the K562-FasL clone induced lysis of L1210-Fas in a dose dependent fashion, although no specific lysis was detected with vector-transfected K562 cells. The PC cell line ALVA-31 behaved similarly (Fig. 1B), with cell lysis reaching 63% at the 10:1 ratio of effectors to targets. A summary of the results with all 8 target cell lines is presented in Table I. The cell lines are listed in order of descending apoptotic potentials as observed in the cytotoxicity assay. Also presented in the third column of Table I, are the results from previous studies using an agonistic anti-Fas IgM antibody (Hedlund 1998). These results demonstrate a markedly enhanced activity of membrane-expressed hFasL versus the soluble IgM antibody.

An interesting feature of the prostate is that it is believed to escape immune surveillance to some extent "... because it lacks afferent lymphatics and because of the immunosuppressive properties of seminal fluid" (McClinton 1990). In fact, the normal prostatic epithelium has been found to coexpress Fas and FasL, as indicated by studies with both mouse (Leithauser 1993; French 1996) and human (Xerri 1997) prostatic tissue. Therefore, it may be more relevant to test the sensitivities of the PC cell lines to internally expressed FasL through transgene methods of gene expression, rather than to FasL-expressing lymphocytes. Given that FasL expression is expected to induce apoptosis, the development of stable transfectants is unlikely to be feasible. Instead, we chose to develop an adenoviral system of gene transduction. Adenovirus entry has

been shown to be highly efficient in cells that express the integrin family of adhesion molecules (Wickham 1993; Huang 1996) and this is a common feature of many PC cell lines (Witkowski 1993; Haywood-Reid 1997).

The Adenovirus-FasL Construct and Propagation in 293/crmA Cells. The mFasL cDNA with a CMV promoter was inserted into the mini-cassette region of a replication deficient human adenovirus 5 construct. Initial attempts to propagate the virus in 293 cells, however, resulted in early death of the cells before significant viral titers could be achieved (data not shown). Greatly reduced viral titers were also reported by Muruve et al. in a similar attempt (Muruve 1997). We suspected that 293 cells may be undergoing Fas-mediated apoptosis before significant viral replication occurred. To test this hypothesis, 293 cells were analyzed for Fas expression by flow cytometric immunofluorescence (Fig. 2A). The mean Fas fluorescence was 4.9 fold greater than nonspecific fluorescence. This value is relatively high compared to other cell lines that we have tested using the same method, and is similar to the level of Fas expressed by CEM cells (Hedlund 1998). Subsequent cytotoxicity experiments indicated that 293 cells are extraordinarily sensitive to the lytic effects of K562-FasL (Fig. 2B), with complete lysis occurring at the 10:1 effector to target ratio. Together, these data support the hypothesis that 293 cells are undergoing Fas-mediated apoptosis during our attempts to produce adeno-FasL virus. To overcome this problem, 293 cells were stably transfected with a cowpox crmA cDNA construct, that was previously shown to prevent Fas and TNF-mediated apoptosis by inhibiting the Caspase family of cysteine proteases (Tewari 1995; Tewari 1995). The resulting 293/crmA transfectants were almost entirely resistant to K562-FasL (Fig. 2C) and effectively propagated the adeno-FasL construct, yielding viral titers of approximately $5x10^8$ pfu/ml.

Efficiency of Adenovirus Gene Transduction in PC Cells. To be sure that adenovirus-mediated gene expression could be achieved efficiently in the PC cells, each cell line was transduced with an adenoviral construct containing a modified jellyfish green fluorescent protein (GFP) cDNA. Cells were incubated with adeno-GFP, were washed, and after 24h of incubation, were analyzed for positive fluorescence by flow cytometry. A summary of these results is shown in Table II. GFP expression was very efficient in six of the seven cell lines, with greater than 90% of the cells being fluorescent. However, the mean GFP fluorescence varied considerably among these same cell lines. LNCaP was the only cell line that showed relatively poor adenoviral gene transduction, as measured by both the percent positive cells (61%), and the relatively low fluorescence intensity of GFP-transduced cells (13 fold brighter than control cells). These data indicate that the adenoviral system is effective in at least six of the seven PC cell lines.

Effects of Adeno-FasL Transduction on PC Cell Growth. To determine how the PC cell lines respond to internally expressed FasL, the growth of cell monolayers was measured for 1 week after transduction with either adeno-FasL or adeno-GFP as a control. As shown in Fig. 3, ALVA-31 cells that were treated with adeno-GFP show logarithmic growth as they approach confluency. A phase-contrast photograph was taken at day 4 and is shown to the right of the growth curve in Fig. 3. The adeno-GFP did not increase apoptosis above basal levels as was confirmed by the lack of nuclear fragmentation after staining cells with propidium iodide and Hoechst 33342 and observing under a fluorescent microscope (data not shown). In contrast ALVA-31 cells that were treated with adeno-FasL were nearly completely apoptotic within 24-48 h (Fig. 3) and the few remaining cells failed to resume exponential growth over the course of this assay. These data support the cytotoxicity results, suggesting that ALVA-31 cells are far more sensitive to the natural FasL protein than they are to anti-Fas antibody.

Furthermore, this cell line appears to be more sensitive to internally expressed FasL than that presented by the K562-FasL transfectants.

The short-term growth of other PC cell lines after adenoviral transduction are shown in Fig. 4. The cell line PPC-1 behaves similarly to ALVA-31 in that FasL transduction nearly obliterates the entire population of cells, and positive growth is not detected over the course of the assay. FasL transduction was to varying extents less effective on JCA-1, PC-3 and TSU-Pr1 cell growth in this *in vitro* assay. Interestingly, TSU-Pr1 is far more sensitive to FasL when it is presented by the K562-FasL transfectant than when it is internally expressed. The reasons for this discrepancy are not clear, however the phenomenon may have *in vivo* significance in terms of apoptotic resistance of metastatic prostate cancers that may potentially respond to immunotherapy with activated T-lymphocytes. The PC cell lines DU 145 and LNCaP yielded inconsistent results in the short-term growth assays with repeated trials (data not shown). For these reasons, we are currently unable to draw any conclusions regarding the sensitivities of these two cell lines to internally expressed FasL.

Affects of Adeno-FasL on Prostate Tumor Growth In Vivo. Although the ALVA-31 cell line appears to be quite sensitive to apoptotic induction by adeno-FasL, it is not clear if the few remaining cells would actually be capable of regenerating over a longer period of time, and if the cells would behave similarly in an *in vivo* environment after FasL transduction. To address these issues, a preliminary experiment was initiated to compare the growth of ALVA-31 cells with and without adeno-FasL after 6 weeks of growth intradermally in *Nu/Nu* mice. Untreated ALVA-31 cells produced tumors in 9 of 12 injection sites. However, ALVA-31 cells that were infected with adeno-FasL (10 pfu/cell) prior to injection failed to produce tumors in any of 4 sites. Also of interest in this trial experiment is that the established control tumors could not be eradicated by later injection of adeno-FasL virus (10^7 pfu/tumor). Although localized apoptosis was

apparent in tissue sections near the injection sites (data not shown), the virus may not have been sufficiently dispersed within the tumor to cause significant regression. This preliminary experiment raised several other important questions. For example, is the lack of tumor growth due specifically to FasL expression, or is the same effect observed with adeno-GFP? Also, what are the *in vivo* effects of adeno-FasL in a prostate tumor cell line that appeared resistant *in vitro*? To address these issues, a second experiment was carried out in mice using the PC cell line TSU-Pr1, whose growth was not inhibited by either adeno-GFP or adeno-FasL *in vitro*.

TSU-Pr1 were pre-infected with adeno-GFP or -FasL as discussed above for the ALVA-31 cell line, and cells were injected intradermally into *Nu/Nu* mice. After 9 days, when control tumors were established, 4 control tumors were injected with free adeno-GFP or -FasL virus. One animal was then sacrificed 24 h later for histologic analysis. Calipers were used to measure the length and width of each tumor at days 9 and 18. The remaining animals were sacrificied after a total of 18 days. As shown in Table III, there was no significant difference between the mean sizes of control and adeno-GFP-infected TSU-Pr1 tumors either at day 9 or 18. In contrast, adeno-FasL tumors were significantly smaller and no change in tumor size was detected between days 9 and 18. Histologic analyses of these tumors revealed several surprizing findings. First, adeno-FasL tumors, although small, appeared viable ruling out the possibility that scar tissue had completely replaced the tumor cells. Histologic analyses also revealed that both adeno-FasL and -GFP treated tumors had extensive neutrophil infiltration (data not shown). This suggests that the initial infiltration of neutrophils is induced nonspecifically by adenoviral infection. Furthermore, this infiltration is not in itself responsible for the regression/rejection of FasL-expressing tumors, as adeno-GFP tumors were as large and as viable as control tumors. It was observed, however, that the neutrophils in the adeno-FasL tumors often appeared apoptotic (data not shown). Thus, it is plausible that once the neutrophils are recruited to the FasL-expressing

tumor, they undergo apoptosis in response to FasL, and may potentiate a greater inflammatory response that indirectly suppresses TSU-Pr1 growth. Further experimentation will be necessary to better define the role of neutrophils in mediating the rejection/regression of tumor cells that are not intrinsically sensitive to adeno-FasL mediated apoptosis.

Perspective. During the course of the current study, Arai et al. addressed similar questions regarding the effects of FasL expression on the growth of colon cancer cell lines in mice (Arai 1997). Overall, their findings strongly support the therapeutic potential of FasL transgene expression in causing the regression of tumors. Furthermore, their data suggest that this favorable response is not only observed in cell lines that undergo apoptosis in response to FasL expression *in vitro*. In fact, tumor regression also occurs in a Fas-negative (and FasL-insensitive) cell line due to the induction of an inflammatory response with neutrophil infiltration. These results are at least in partial agreement with our findings in prostate cancer cells. And whether or not this effect is directly due to the actions of neutrophils, it follows that this type of gene therapy might be most useful for the treatment of slow-growing cancers, such as those of prostatic origin. It has been estimated that the doubling times of late stage prostate cancers are typically near 2 years (Schmid 1993). Therefore, a therapeutic strategy that can even modestly extend this doubling time, could extend a patient's life by a number of years, especially if initiated soon after diagnosis.

Conclusions

The data presented in the current study indicate that several human PC cell lines are significantly more sensitive to FasL-mediated apoptosis than was originally reported with the use of agonistic anti-Fas IgM antibodies. Furthermore, the majority of these cell lines respond maximally to FasL when it is expressed internally via the adenoviral system. This method of internal expression may better represent what occurs in the prostate *in vivo* since the prostatic epithelium has been shown to coexpress Fas and FasL (Leithauser 1993; French 1996; Xerri 1997). Our preliminary *in vivo* experiments, in conjunction with the recent findings of Arai et al. (Arai 1997) suggest therapeutic potential for FasL transgene expression in treating cancer patients. Given the prevalence of prostate cancer and the limited effectiveness of available therapies, further research in this area seems warranted.

Acknowledgements

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TABLE I. Specific Lysis of Human Prostate Cancer Cell Lines By K562-FasL Transfectants at a 10:1 Effector to Target Ratio: A Comparison With Cell Lysis Achieved Using an Anti-Fas IgM Antibody in Previous Studies*

TARGET CELL LINE	% LYSIS BY K-562 FASL CLONE	% LYSIS BY ANTI-FAS IgM ANTIBODY*
L1210-FAS	80 ± 10	(not determined)
ALVA-31	63 ± 5	28%
TSU-Pr1	52 ± 13	undetectable
PPC-1	48 ± 1	10%
JCA-1‡	47 ± 6	10%
LNCaP	27 ± 3	undetectable
DU 145‡	21 ± 2	undetectable
PC-3	19 ± 5	undetectable

* Previously published data (Hedlund 1998)
‡ JCA-1 and DU 145 showed slight but statistically significant lysis by the vector-transfected K562 clone (14% ± 1, and 4% ± 0.2, respectively).

TABLE II. Efficiency of Adeno-GFP Expression in 7 PC Cell Lines. GFP fluorescence was measured both by the percent positive cells and the fold increase in mean fluorescent intensity as compared to untreated cells.

Cell Line	% GFP Positive Cells	Fold Increase in Mean Fluorescence
ALVA-31	99.6	118
JCA-1	98.7	172
PPC-1	98.1	235
DU 145	97.2	813
TSU-Pr1	93.2	55
PC-3	92.2	129
LNCaP	61.2	13

TABLE III. The Effects of Adeno-FasL on Growth of TSU-Pr1 Cells in Nude Mice. Cells were untreated (control), or pre-infected with Adeno-GFP or Adeno-FasL. For comparison, several established control tumors were injected at day 10 with free Adeno-GFP or Adeno-FasL virus.

[illegible]	[illegible]	[illegible]
Control	9.7 ± 1.9	21 ± 10.3
Adeno-GFP	12.6 ± 2.3	27.5 ± 4.3
Adeno-FasL	2.3 ± 0.9	2.3 ± 0.9
Injected Adeno-GFP	-	31 ± 3.9
Injected Adeno-FasL	-	23 ± 9.2

Figure Legends

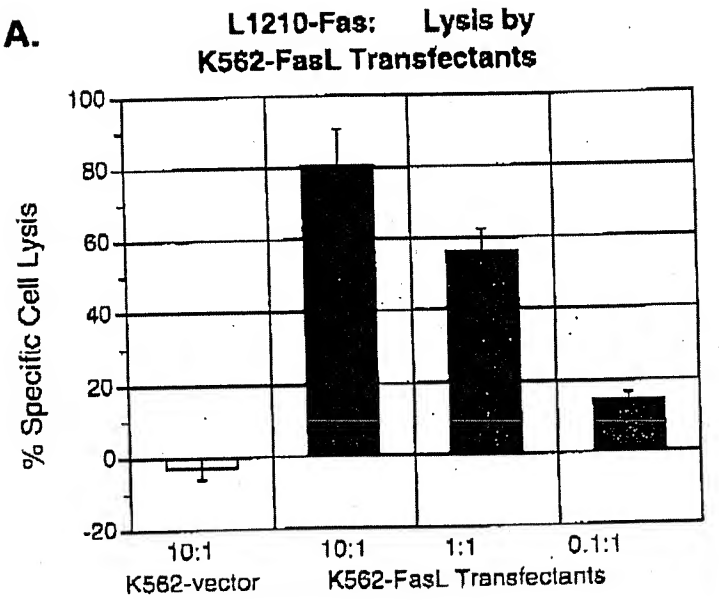
Figure 1. The dose-dependent lysis of (A) L1210-Fas or (B) ALVA-31 target cells by a K562 clone that stably expresses hFasL. Lysis was measured using a ^{51}Cr-release assay after an 18 h coincubation of target and effector cells. Each value represents the mean of triplicate samples ± S.D.

Figure 2. (A) Flow-cytometric analysis of Fas expression by 293 cells. Surface Fas expression was detected using an FITC-conjugated mouse antihuman Fas IgG1 antibody (black peak). Nonspecific fluorescence was estimated using an isotype-matched control antibody directed against a non-mammalian protein (gray peak). (B) Specific lysis of 293 cells or (C) 293/crmA transfectants by K562-FasL as measured by the ^{51}Cr-release assay. Each value represents the mean of triplicate determinations ± S.D.

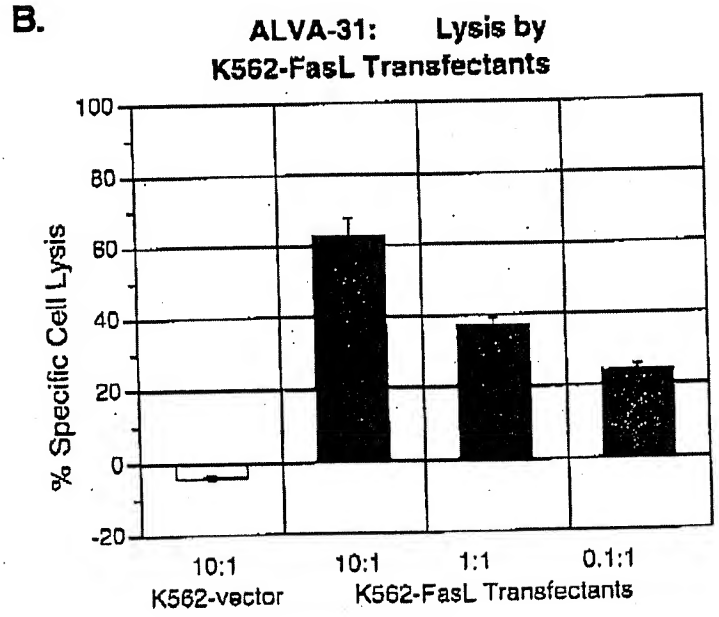
Figure 3. Growth of ALVA-31 cells after adenoviral gene transduction of FasL or GFP. Cell monolayers were harvested at the designated time points for DNA quantitation to measure changes in growth. Each value represents the mean of triplicate samples ± standard error. To the right of each condition is a phase-contrast image of ALVA-31 cells as they appeared at Day 4 of the growth assay.

Figure 4. Growth of four prostate cancer cell lines after adenoviral transduction of GFP or FasL. The experimental design follows that described for Fig. 3.

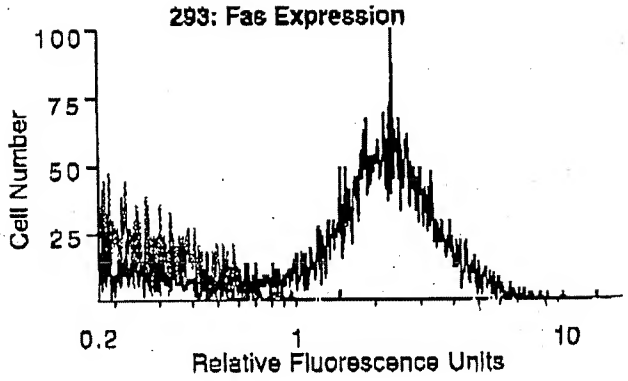
A.


L1210-Fas: Lysis by
K562-FasL Transfectants
% Specific Cell Lysis
100
80
60
40
20
0
-20
10:1
K562-vector
10:1
1:1
0.1:1
K562-FasL Transfectants

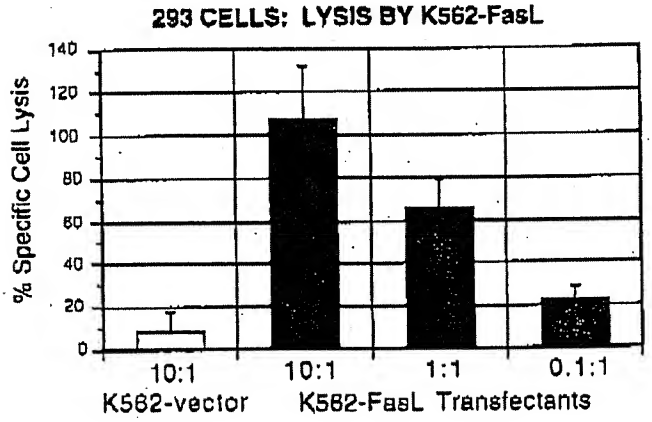
B.


ALVA-31: Lysis by
K562-FasL Transfectants
% Specific Cell Lysis
100
80
60
40
20
0
-20
10:1
K562-vector
10:1
1:1
0.1:1
K562-FasL Transfectants

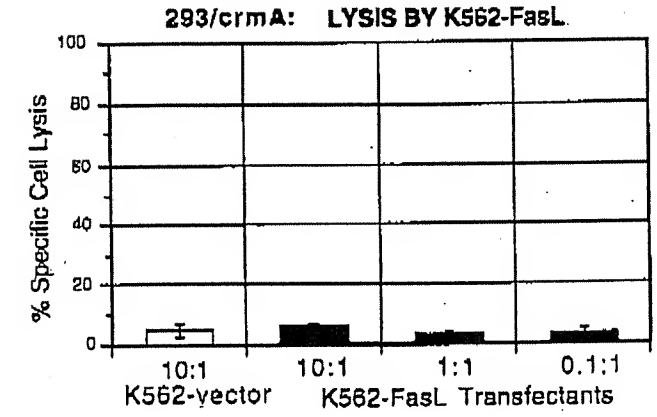
A.

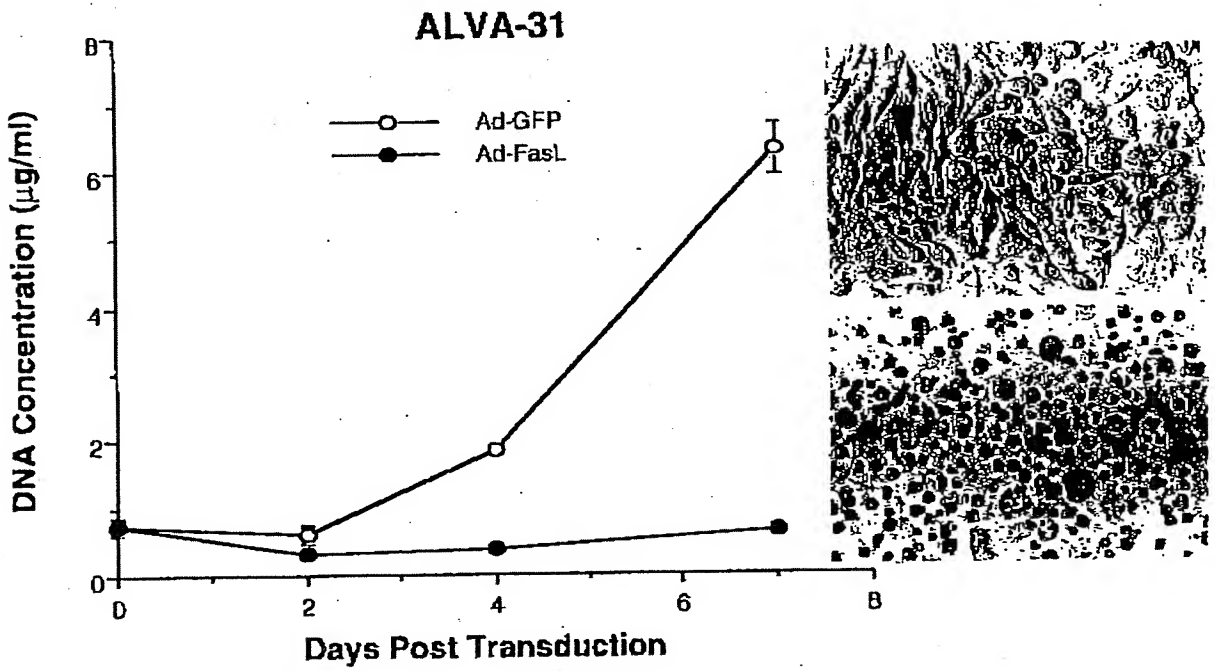

293: Fas Expression
100
75
50
25
Cell Number
0.2
1
10
Relative Fluorescence Units

B.


293 CELLS: LYSIS BY K562-FasL
140
120
100
80
60
40
20
0
% Specific Cell Lysis
10:1
10:1
1:1
0.1:1
K562-vector
K562-FasL Transfectants

C.


293/crmA: LYSIS BY K562-FasL
100
80
60
40
20
0
% Specific Cell Lysis
10:1
10:1
1:1
0.1:1
K562-vector
K562-FasL Transfectants


ALVA-31
Ad-GFP
Ad-FasL
DNA Concentration (µg/ml)
0
2
4
6
8
0
2
4
6
8
Days Post Transduction

GROWTH OF PROSTATE CANCER CELL LINES AFTER ADENOVIRAL TRANSDUCTION OF GFP OR FAS LIGAND